Peroxidative Modification of a Membrane Protein. Conformation-Dependent Chemical Modification of Adenine Nucleotide Translocase in Cu^{2+}/*tert*-Butyl Hydroperoxide Treated Mitochondria[†]

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ABSTRACT: Peroxidative treatment of rat heart mitochondria results in a gradual increase of the apparent molecular weight of the adenine nucleotide translocase (ANT) by up to 1.2 kDa. ANT isolated from mitochondria treated with 1 mM *tert*-butyl hydroperoxide and 5−40 μM Cu^{2+} for 1 h at 37 °C exhibited a progressive loss of lysine, cysteine, arginine, and valine residues compared to native ANT. *N*-Ethylmaleimide, dithiothreitol, and the specific inhibitor of ANT, carboxyatractyloside (CAT), inhibited the peroxidation-induced molecular weight shift without inhibiting lipid peroxidation, which is believed to be the primary cause of the observed ANT modification. Bongkrekic acid, which stabilizes ANT in a conformation different from that brought about by CAT, did not inhibit the ANT molecular weight shift. Dithiothreitol, as well as CAT, was found to protect ANT against most of the losses of amino acid residues, indicating that alteration of sulfhydryl residues is required for chemical modification of, not only cysteine, but also lysine, arginine, and valine. We conclude that the peroxidative modification of ANT is conformation-dependent and involves chemical modification of cysteine as a critical step.

It has been proposed that excessive levels of reactive oxygen species are involved in the etiology of diseases such as atherosclerosis, ischemia-reperfusion injury, and cancer, as well as aging (Ames et al., 1993; Halliwell & Gutteridge, 1990; Marx, 1987; Stadtman, 1992). Direct metal-catalyzed oxidation of proteins may explain some of the deleterious effects of oxygen free radicals (Davies, 1988; Dean et al., 1993; Gordillo et al., 1989; Stadtman & Oliver, 1991). Alternatively, chemical modification of proteins may be due to reactions with products formed during the peroxidative decomposition of lipids containing polyunsaturated fatty acids (Esterbauer et al., 1991; Frankel, 1984; Kikugawa & Beppu, 1987; Slater, 1984). Information on the latter type of reactions is quite limited and based largely on the study of relatively simple model reactions of specific molecules in solution [reviewed by Esterbauer et al. (1991) and Kikugawa and Beppu (1987)]. It is therefore of interest to establish possible chemical modifications of proteins in complex biological systems resembling more closely *in vivo* conditions. In this context, we have determined lipid and protein alterations in subcellular preparations of rat heart with the goal of determining primary molecular targets of peroxidative damage. We have found that several different peroxidative treatments of myocardial membranes, including the generation of oxygen free radicals and more direct induction of lipid peroxidation, including lipoxygenase treatment, preferentially affect a protein of 28 kDa (Parinandi et al., 1991) which was later identified as the mitochondrial adenine nucleotide translocase (ANT)[1] (Zwizinski & Schmid, 1992). The protein's peroxidative modification was characterized as a gradual increase of its apparent molecular weight by up to 1.2 kDa, followed by polymerization and fragmentation reactions under stronger peroxidative treatment (Girón-Calle et al., 1994). Reaction with lipid peroxidation products rather than direct oxidation is most likely the cause of the molecular weight increase (Girón-Calle et al., 1994; Parinandi et al., 1990, 1991; Zwizinski & Schmid, 1992), and the peroxidative modification of ANT provides a good model for the study of free radical-induced protein modification in a complex biological environment such as the mitochondrial inner membrane.

Modified ANT species have now been isolated from rat heart mitochondria subjected to different degrees of peroxidative treatment in order to determine the chemical modification of amino acid residues. The effects on ANT modification of the specific inhibitors carboxyatractyloside and bongkrekic acid, and other reagents, have also been determined and are explained in the context of available information regarding the structure and translocational states of membrane-bound ANT.

EXPERIMENTAL PROCEDURES

Chemicals. Carboxyatractyloside (CAT), dithiodiglycolic acid, α-aminobutyric acid, and diethyl ethoxymethylenemalonate were obtained from Sigma (St. Louis, MO). Bongkrekic acid (BKA) was a gift of Prof. R. Krämer (Jülich, Germany).

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[1] Abbreviations: ANT, adenine nucleotide translocase; HPLC, high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBARS, thiobarbituric acid reactive substances; *t*-BuOOH, *tert*-butyl hydroperoxide; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; CAT, carboxyatractyloside; BKA, bongkrekic acid; P5P, pyridoxal 5-phosphate; MDA, malondialdehyde.

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Preparation and Incubation of Mitochondria. Male Sprague-Dawley rats weighing approximately 250 g were used for preparation of rat heart subsarcolemmal mitochondria essentially as described by Palmer et al. (1977, 1981). The preparations were washed twice with incubation buffer (150 mM NaCl, 20 mM Hepes, pH 7.4), frozen in liquid nitrogen, and kept at −70 °C. Mitochondria were pelleted once more immediately before use. Incubations were carried out by shaking for 1 h at 37 °C in incubation buffer at a concentration of 1.5 mg of protein/mL. The pH was adjusted to 6.7 for the experiments involving addition of BKA and the corresponding controls. Peroxidation was induced by addition of 1 mM *t*-BuOOH and different concentrations of $CuCl_2$ ranging from 5 to 40 μM and stopped by addition of EDTA in a final concentration of 1 mM and cooling down on ice. In some cases, preincubation with some reagents for 5 min at room temperature was carried out before peroxidative treatment. Aliquots were taken immediately for analysis and ANT purification.

ANT Purification. Native and peroxidatively modified ANT was purified by extraction in a buffer containing Triton X-100 and successive hydroxylapatite and Celite column chromatography as described previously (Zwizinski & Schmid, 1992). For amino acid analysis, the proteins were precipitated from the SDS-containing buffer (Zwizinski & Schmid, 1992) by addition of 3 volumes of acetone and 10% (w/v) trichloroacetic acid (Bogner et al., 1986). After the solution was left overnight in the freezer, pellets were obtained by centrifugation and washed once with acetone/water (1:1) and twice with water.

Amino Acid Analysis. For acid hydrolysis the protein samples were treated with 6 N HCl, 0.1% (v/v) phenol, and 0.1% (w/v) dithiodiglycolic acid for 22 h at 110 °C in vials purged with nitrogen. Dithiodiglycolic acid was added to the hydrolysis solution in order to stabilize cysteine and cystine residues by forming the corresponding mixed disulfide derivative (Hoogerheide & Campbell, 1992). Precolumn derivatization of the acidic hydrolysates with diethyl ethoxymethylenemalonate and reverse phase HPLC analysis using α-aminobutyric acid as an internal standard were carried out essentially as described by Alaiz et al. (1992). Cysteine plus half-cysteine was determined as the dithiodiglycolic acid−diethyl ethoxymethylenemalonate derivative of cysteine, which has a retention time between those of serine and histidine in HPLC.

Other Methods. Thiobarbituric acid reactive substances (TBARS), expressed as nmol of MDA-equivalent/mg of protein, using 1,1,3,3-tetramethoxypropane as an internal standard, were routinely determined in aliquots of the incubations (Huber et al., 1975). This assay provides a sensitive determination of lipid peroxidation that correlates very well with decomposition of polyunsaturated fatty acids in peroxidized mitochondria (Girón-Calle et al., 1994). SDS−PAGE was carried out following an established method (Laemmli, 1970), using gels containing 16% (w/v) acrylamide and 0.1% (w/v) bisacrylamide and sample buffer containing 60 mM dithiothreitol as reducing agent (Girón-Calle et al., 1994). After electrophoresis, gels were stained with Coomassie brilliant blue R-250 following the procedure described earlier (Zwizinski & Schmid, 1992) or according to Chrambach et al. (1967). Total and purified proteins were determined by the method of Lowry et al. (1951) as previously described (Zwizinski & Schmid, 1992). The accuracy of this method to determine both native and modified ANT was checked by comparing it with quantification by amino acid analysis. Phosphorus was measured according to Bartlett (1959) in ANT protein purified and washed as described above.

Table 1: Amino Acid Composition of Native Rat Heart ANT Determined by Amino Acid Analysis of Acid Hydrolysates and Compared to the Composition Deduced from Nucleotide Analysis by Shinohara et al. (1993)

	amino acid analysis[a]	deduced from cDNA clone[b]
Asx[c]	21.5 ± 0.6	23
Glx[d]	21.8 ± 0.7	20
Ser	17.0 ± 0.4	17
Cys	4.1 ± 0.2	4
His	3.1 ± 0.1	3
Gly	31.4 ± 0.3	31
Thr	13.2 ± 0.2	10
Arg	15.7 ± 0.4	17
Ala	31.2 ± 0.5	31
Pro	7.8 ± 1.7	7
Tyr	12.9 ± 0.7	13
Val	21.6 ± 0.6	24
Met	7.9 ± 0.5	8
Ile	16.1 ± 0.3	18
Leu	27.2 ± 0.3	23
Phe	18.7 ± 0.3	20
Lys	21.7 ± 0.4	24
Trp[e]		5
total	293	298

[a] Data represent average ± standard deviation of the analysis of six different protein preparations as described in Materials and Methods. Here and in the following figures the raw amino acid analysis, expressed as relative % mol composition, was based on a total of 293 residues per molecule (i.e., 298 residues deduced by cDNA analysis minus 5 residues of trypotophan destroyed during acid hydrolysis). [b] Amino acid sequence was deduced by nucleotide analysis of a cDNA clone isolated from libraries constructed from rat heart mRNA (Shinohara et al., 1993). This is the heart-skeletal muscle ANT type 1 (ANT1). Post-translational modifications are known to occur in beef heart ANT (Shinohara et al, 1993; Klingenberg & Nelson, 1994), and most likely in rat heart ANT as well. [c] Asn + Asp. [d] Gln + Glu. [e] Trp is lost during acidic hydrolysis.

RESULTS

Amino Acid Composition of Native and Peroxidatively Modified ANT. The amino acid composition of rat heart ANT, as determined by amino acid analysis of acid hydrolysates, is listed in Table 1 and compared to the composition deduced from nucleotide analysis of a cDNA clone (Shinohara et al., 1993). The 28 kDa protein susceptible to peroxidative modification (Figure 1) had earlier been identified as ANT on the basis of its chromatographic behavior and response to the specific inhibitor CAT (Zwizinski & Schmid, 1992). The match shown in Table 1 confirms this identification.

ANT was purified from mitochondria that had been incubated with 1 mM *t*-BuOOH and 5, 10, 20, and 40 μM Cu^{2+} as a free radical generating system (Figure 1). Presumably, the main effect of treatment with *t*-BuOOH/Cu^{2+} is the induction of autoxidation of endogenous polyunsaturated fatty acids by the *tert*-butyl alkoxyl radical (Bindoli, 1988; Girón-Calle et al., 1994; Masaki et al., 1989) rather than through generation of reactive oxygen species as occurs by treatment with Fe^{2+}/ascorbate (Halliwell & Gutteridge, 1990; Parinandi et al., 1991). Treatment of individual proteins in solution (lysozyme, bovine serum albumin) with 1 mM *t*-BuOOH and 10 μM $CuCl_2$ in the absence of lipids did not cause any alteration as assessed by SDS−PAGE and amino

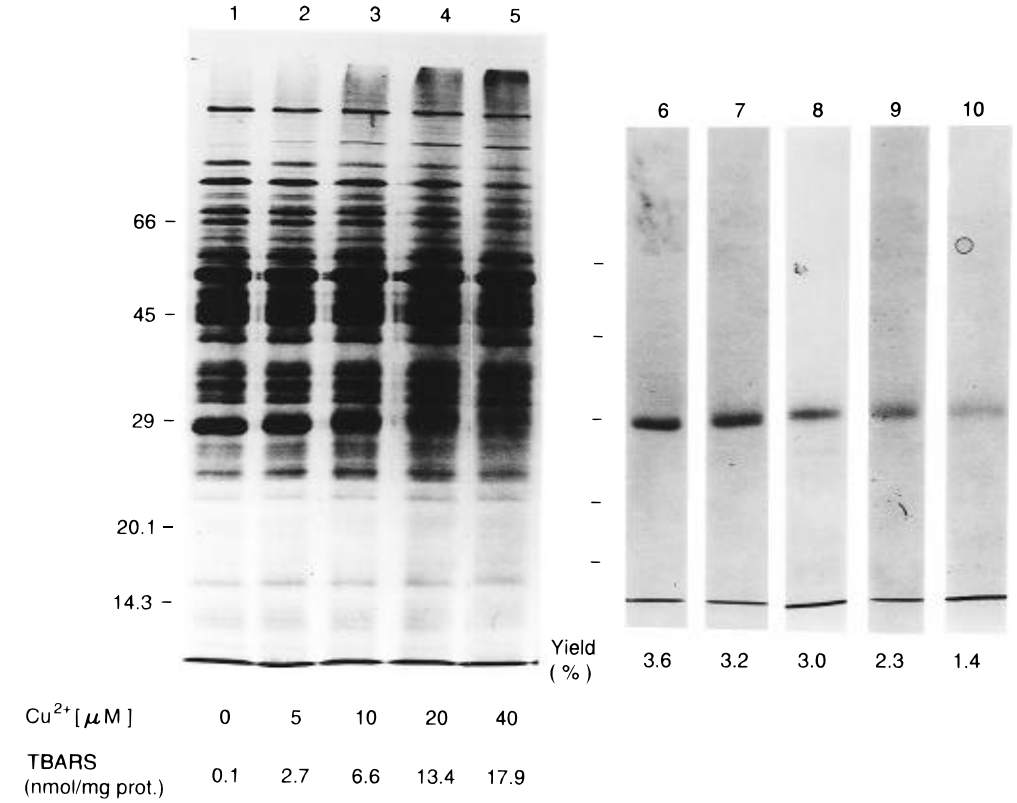

FIGURE 1: SDS−PAGE of mitochondria treated with 1 mM *t*-BuOOH and increasing concentrations of Cu^{2+}, and of ANT species isolated from these samples. Each line represents 11 μg of total (lanes 1−5) or 0.9−0.4 μg of purified (lanes 6−10) protein. Values for TBARS generated during the incubation and yields of protein purification (% of initial mitochondrial protein) for a typical experiment are shown.

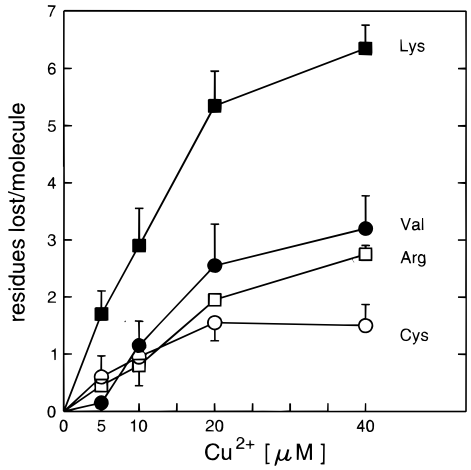

FIGURE 2: Amino acid residues lost in ANT isolated from mitochondria treated with *t*-BuOOH (1 mM) and Cu^{2+} (5, 10, 20, and 40 μM) (SDS−PAGE of these species is shown in Figure 1). Error bars represent the standard deviation of 6 (10 μM Cu^{2+}) or 3 (5, 20, and 40 μM Cu^{2+}) experiments.

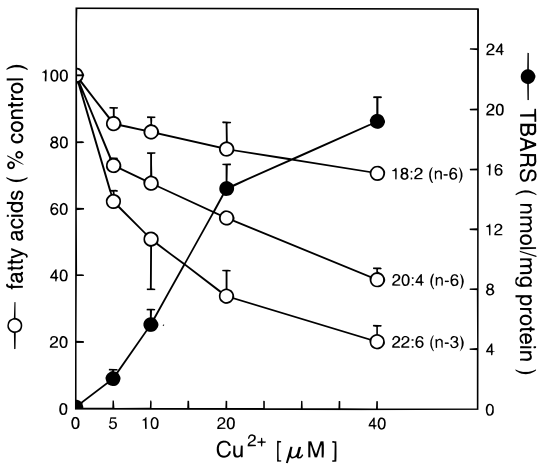

FIGURE 3: Peroxidative decomposition of lipids in *t*-BuOOH/Cu^{2+}-treated mitochondria as assessed by TBARS and fatty acid analysis. Data represent average ± standard deviation of three experiments, modified from Girón-Calle et al. (1994).

acid analysis (Girón-Calle et al., unpublished observations). Peroxidative treatment of mitochondria with 1 mM *t*-BuOOH and up to 20 μM Cu^{2+} induces a gradual increase of the apparent molecular weight (Figure 1) that characterizes the early stages of ANT peroxidative modification (Girón-Calle et al., 1994). Electrophoretically pure ANT species obtained from these preparations (Figure 1) were precipitated, washed, and subjected to acid hydrolysis and amino acid analysis in order to assess chemical modification of the protein (Figure 2). Lysine, arginine, cysteine, and valine residues were found to be affected by the peroxidative treatment. Increasing strength of the treatment caused increasing losses of these residues. This process roughly paralleled TBARS generation and the molecular weight increase of ANT (Figures 1 and 3). The equivalent of up to about six residues of lysine, three residues of arginine and valine, and one residue of cysteine are readily susceptible to peroxidative modification. This constitutes 27, 12, 14, and 25% of the total lysine, arginine, valine, and cysteine residues in the protein, respectively. This level of modification is only achieved with strong treatments, but even the mildest treatment tested, generating only 2 nmol of TBARS/mg of protein, modified the equivalent of about two residues of lysine and one-half residue of both arginine and cysteine.

No new peaks representing modified amino acids (Alaiz & Girón, 1994a; Szweda et al., 1993) appeared in the chromatograms derived from modified ANT (not shown). However, some peaks in the region representing certain polar

Table 2: Effect of Different Inhibitors and Reagents on ANT Modification and Lipid Peroxidative Decomposition in *t*-BuOOH/Cu^{2+}-Treated Mitochondria[a]

additions in		TBARS	ANT	
preincubation[b]	incubation	(nmol/mg of protein)	modification: +/−	no. of expts
na	na	9.06 ± 1.59	+	9
na	BHT	0.94 ± 0.13	−	5
na	mannitol	9.27 ± 0.81	+	3
na	DTT	9.42 ± 2.94	−	6
na	NEM	7.45 ± 1.34	−	6
P5P (10 mM)	na	5.13	+	1
np	P5P (0.1 mM)	3.89	+	1
np	P5P (20 μM)	5.76	+	1
np	CAT	9.80 ± 1.76	−	5
CAT	na	9.38 ± 1.27	−	5
np	BKA	8.40 ± 2.01	+	4
BKA	CAT	7.34 ± 1.07	+	2
CAT	BKA	6.75 ± 1.16	−	2
BKA	DTT	6.07 ± 2.90	−	2
BKA	BHT	0.70 ± 0.08	−	2

[a] *t*-BuOOH (1 mM) and Cu^{2+} (10 μM) were added after incubating for 5 min at room temperature with the additions referred to in the table. Mitochondria were pelleted and resuspended between preincubation and incubation. Additions were as follows unless otherwise stated in the table: BHT, 5 mM; mannitol, 10 mM; DTT, 5 mM; NEM, 0.1 mM; CAT, 30 μM; BKA, 60 μM. Other details are given in Materials and Methods. Average ± standard deviation of TBARS is shown. [b] na, no preincubation or no addition during preincubation; np, no preincubation.

amino acids exhibited an increase in area that roughly paralleled the loss of other amino acids (lysine, cysteine, arginine, and valine), indicating possible overlapping of peaks corresponding to modified and unmodified amino acids.

Phosphorus Content of Peroxidatively Modified ANT. In order to check whether peroxidative ANT modification involves adduct formation with peroxidized phospholipid(s), duplicate aliquots (0.1−0.3 mg) of ANT preparations that had been treated with 1 mM *t*-BuOOH and 10 or 20 μM Cu^{2+} were assayed for phosphorus by the Bartlett (1959) procedure. They were found to contain approximately 2 (1.7, 1.8) and 3 (2.9, 2.6) mol of P/mol of ANT, respectively, whereas native ANT contained none. This supports, but does not prove, the hypothesis that products of lipid peroxidation, still carrying the glycerophosphate backbone, were covalently attached to the modified ANT.

Effect of Butylated Hydroxytoluene, Mannitol, N-Ethylmaleimide, and Dithiothreitol on ANT Modification. Treatment with 1 mM *t*-BuOOH and 10 μM Cu^{2+} (Figure 1, lane 3) was chosen to assess the effect of several reagents and inhibitors in the peroxidative modification of ANT as determined by SDS−PAGE. The results of these experiments are summarized in Table 2. A "positive" ANT modification refers to the occurrence of a molecular weight shift and a decrease in band intensity such as those shown in Figures 1 and 4 (Figure 1, lanes 3 and 8; Figure 4, lanes 2 and 6). A "negative" result means that the band corresponding to ANT remained essentially unaltered (Figure 1, lanes 1 and 6; Figure 4, lanes 1, 3, 4, 5, 7, and 8). Here we establish a qualitative distinction between molecular weight-shifted and non-molecular weight-shifted ANT as a convenient way to assess ANT modification. In all the cases in which ANT species were isolated and their amino acid composition determined, the same correlation between most of the amino acid losses and molecular weight shift was found. The quantification of the molecular weight-shifting process, which is associated with a decrease in the intensity of the Coomassie Blue-stained ANT band, has been published previously (Girón-Calle et al., 1994).

Previous data indicate that butylated hydroxytoluene (BHT) and dithiothreitol (DTT) inhibited the peroxidation-induced molecular weight shift of ANT when total myocardial membranes were treated with Fe^{2+}/ascorbate, whereas mannitol did not have any protective effect (Parinandi et al., 1991). This observation has now been reproduced in mitochondrial preparations treated with *t*-BuOOH/Cu^{2+}. The permeant sulfhydryl reagent *N*-ethylmaleimide also had a protective effect. The effects of BHT, an inhibitor of lipid peroxidation, and mannitol, a hydroxyl radical scavenger, are consistent with the hypothesis that reaction with lipid peroxidation products is the primary cause of ANT peroxidative modification (see also Table 2, TBARS). More interesting is the observation that DTT and NEM exhibit a protective effect without preventing lipid peroxidation. This points to cysteine residues as being involved in the peroxidative modification of the protein leading to the increase in its apparent molecular weight. Treatment with different concentrations of the amino group reagent pyridoxal 5-phosphate (Bogner et al., 1986; Lê Quôc & Lê-Quôc, 1988) did not prevent the ANT molecular weight shift (Table 2).

Effect of Carboxyatractyloside and Bongkrekic Acid on ANT Peroxidative Modification. CAT and BKA are specific inhibitors that fix ANT in two different conformational states depending on which side of the inner mitochondrial membrane is faced by the protein's binding site. CAT fixes the protein in the so-called "c-state", with the active site facing the intermembrane space, and BKA fixes the protein in the "m-state", with the binding site facing the mitochondrial matrix. Inhibitors and substrates bind to the same active site which can face only one side of the membrane at a time. This is based on the "single-binding-center gated pore" mechanism, established by Klingenberg and co-workers [reviewed by Klingenberg (1989, 1993) and Klingenberg and Nelson (1994)]. This mechanism has become a paradigm for the mechanism of action of membrane carrier proteins in general. Because CAT protects ANT from peroxidative modification (Zwizinski & Schmid, 1992), it was of interest to investigate the effect of the opposing inhibitor BKA. No protective effect was found for BKA (Table 2). In contrast to CAT whose binding to ANT is ADP-independent in freshly isolated mitochondria, binding of BKA has been described to be ADP-dependent (Aquila et al., 1978), probably due to the carrier being mostly in the c-state. For this reason, the effects of several concentrations of BKA ranging from 15 to 240 μM in the presence of 75 μM ADP were assayed, with the same negative result (data not shown).

Preincubation with BKA before peroxidative treatment in the presence of CAT was carried out in order to prove that BKA binds to ANT during the incubations. Although BKA does not prevent peroxidative modification of ANT as shown by SDS−PAGE, its binding to ANT should fix the protein in the m-state (Aquila et al., 1978, 1982; Aquila & Klingenberg, 1982) and thus prevent the binding and the protective effect of CAT. Indeed, preincubation with BKA prevented the protective effect of CAT during the peroxidative treatment of mitochondria (Table 2). This also indicates that the presence of ADP is not required for BKA binding in our experimental system.

Mitochondrial preparations were also subjected to peroxidative treatment in the presence of BKA and DTT. We

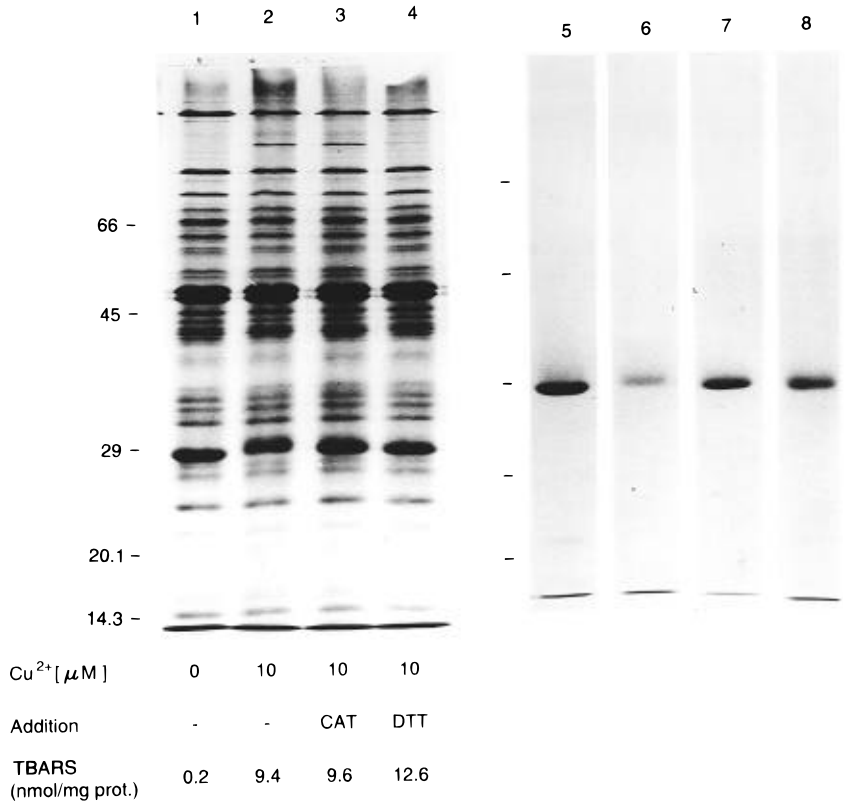

FIGURE 4: SDS−PAGE of mitochondria treated with 1 mM *t*-BuOOH and 10 μM Cu^{2+} in the presence of CAT and DTT (lanes 1−4), and of ANT purified from these same samples (lanes 5−8). Each lane represents 11 μg of total or approximately 1 μg of purified protein.

found that ANT is protected by DTT even when it is fixed in the BKA-bound m-state conformation, which is otherwise susceptible to peroxidative modification (Table 2). This supports the view that the protective effect of DTT, and most likely also of NEM, is due to direct protection of sulfhydryl groups and not to a possible induction of the peroxidation-resistant conformation.

Loss of Amino Acid Residues in ANT Isolated from Mitochondria Peroxidized in the Presence of CAT and DTT. The observation that NEM and DTT prevent the ANT molecular weight shift suggests that this phenomenon is due to chemical modification of cysteine residues, while the bulk of residue modifications (Figure 2) would not have any effect on the apparent molecular weight shift that results from peroxidative treatment. Amino acid analysis of ANT purified from mitochondria peroxidized (1 mM *t*-BuOOH, 10 μM Cu^{2+}) in the presence of CAT and DTT (Figure 4) was carried out in order to compare the protective effect conferred by protection of sulfhydryl groups and by fixing of ANT in a predetermined, apparently peroxidation-resistant, conformation (c-state). Unexpectedly, both additions had essentially the same effect, protecting ANT from most of the residue losses (Figure 5).

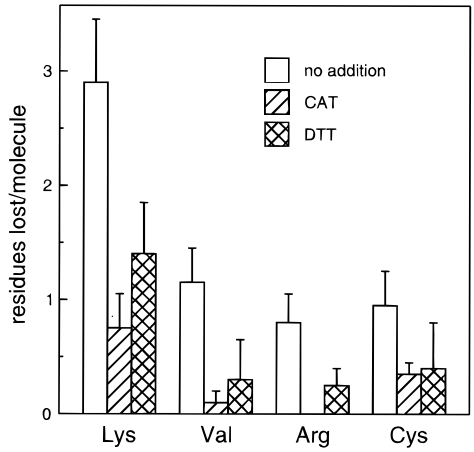

FIGURE 5: Amino acid residues lost in ANT isolated from mitochondria treated with 1 mM *t*-BuOOH and 10 μM Cu^{2+} in the presence of CAT and DTT (shown in Figure 4). Averages ± standard deviations of 6 (no addition) or 3 (CAT, DTT) experiments are represented.

DISCUSSION

The peroxidative modification of ANT *in vitro* which results in a gradual increase of its apparent molecular weight attracted our attention because it begins to occur under relatively mild peroxidative conditions before any other peroxidation-induced changes in the polypeptide profile take place. This suggests a special susceptibility of this protein to peroxidative treatment, which raises the possibility that ANT is a target of peroxidative damage *in vivo.* Because the mitochondrial respiratory chain is a primary source of oxygen free radicals, mitochondrial lipids, proteins, and DNA are considered to be potential targets of peroxidative attack (Ambrosio et al., 1993; Bindoli, 1988; Halliwell & Gutteridge, 1990; Zhang et al., 1990).

The nature of the peroxidative systems that successfully induce the *in vitro* modification of ANT, namely, Fe^{2+}/ascorbate (Parinandi et al., 1991), Cu^{2+}/*t*-BuOOH (Girón-Calle et al., 1994; Parinandi et al., 1991), lipoxygenase, and linoleic acid hydroperoxide (Parinandi et al., 1991), and the protective effect of the lipid peroxidation inhibitor BHT, but not of the hydroxyl radical scavenger mannitol (Parinandi et al., 1991) (Table 2), all support the hypothesis that reaction of the protein with lipid peroxidation products is the primary cause of ANT modification. Although the chemical structure of these products is unknown and is not addressed in the

present study, the presence of phosphorus in the modified ANT supports the hypothesis of covalent attachment of phospholipidic peroxidation products containing the glycerophosphate backbone. At the same time, the more commonly known smaller peroxidation products not containing glycerophosphate are also likely to be involved. In this context it is interesting to note that yeast mitochondria, whose lipids do not contain polyunsaturated fatty acids (Daum, 1985), exhibit both cross-linking and fragmentation of ANT but no shift in its apparent molecular weight as the result of oxidant treatment (C. W. Zwizinski, personal communication). Very recently, reconstituted rat liver ANT was shown to be susceptible to attack by lipid peroxidation products, impairing its activity (Chen et al., 1995).

ANT is known to be tightly bound to six molecules of cardiolipin per dimer of the protein (Beyer & Klingenberg, 1985). This association appears to be common for many carriers and enzymes involved in oxidative phosphorylation (Hoch, 1992). ANT is also rich in lysine residues, some of which are proposed to be in close proximity to cardiolipin molecules assuring tight association of this phospholipid by ionic bonding (Bogner et al., 1986; Klingenberg, 1989). This provides a very favorable environment for modification of the protein by reaction with lipid peroxidation products because lysine is an amino acid especially susceptible to modification by this type of reaction. Rat heart cardiolipin contains a very high proportion (94%) of linoleic acid (Palmer et al., 1981), a diunsaturated fatty acid readily susceptible to free radical attack.

The amino acid analysis of acid hydrolysates used to quantify the chemical modification of ANT in mitochondria subjected to peroxidative treatment supports adduct formation. The losses of lysine, arginine, and cysteine residues may be explained by the susceptibility of amino and sulfhydryl groups to reaction with lipid peroxidation products. Reactions of this kind have been characterized for lipid peroxidation products such as certain aldehydes (Alaiz & Girón, 1994a,b; Chio & Tappel, 1969; Nair et al., 1981; Ohya, 1993; Szweda et al., 1993; Suyama & Adachi, 1979; Uchida et al., 1993; Zamora & Hidalgo, 1994) and hydroperoxides [Gardner et al., 1977; reviewed by Esterbauer et al. (1991) and Kikugawa and Beppu (1987)]. Valine, having an isopropyl group as lateral substituent chain, is not expected to be easily modified by lipid peroxidation products, but it has been shown to be readily altered by irradiation-generated hydroxyl radicals which could mimic direct metal-catalyzed oxidation of proteins (Fu et al., 1995).

The conformation of ANT in the mitochondrial membrane appears to be of critical importance for its susceptibility to peroxidative modification. There is ample evidence showing that exposure of at least lysine, arginine, and cysteine residues is different in the c- and m-states of ANT which are stabilized by the inhibitors CAT and BKA, respectively. Large conformational changes, and not the inhibitor molecule covering residues itself, seem to be the cause of most of the differences in exposure (Aquila et al., 1982; Aquila & Klingenberg, 1982; Bogner et al., 1986). This differential exposure of the residues that are most susceptible to reaction with lipid peroxidation products satisfactorily explains the effect of CAT and BKA on ANT modification. While CAT protects against the peroxidation-induced molecular weight shift and most of the amino acid losses, BKA does not show any protective effect and does not prevent the molecular weight shift. Consequently, ANT peroxidative modification *in vitro* appears to be a conformation-dependent phenomenon.

A second aspect of ANT modification is illustrated by the fact that DTT and NEM also have a protective effect which was shown to be independent of any possible effect on ANT conformation. This clearly points to involvement of cysteine residue(s) in ANT modification. Unexpectedly, both DTT and CAT provide essentially the same protection against most of the residue losses, suggesting that the modification of cysteine may be a critical step in ANT peroxidative modification in the sense that its chemical modification is required for the further alteration of other amino acid residues. Considering that lipid peroxidation is believed to be the primary cause of ANT modification, this could be explained by formation of an adduct between cysteine and peroxidized phospholipid species. By further decomposing in the close proximity of the protein they could provide more reactive molecules (secondary products of lipid peroxidation) capable of modifying other residues, mostly lysine. The molecular weight of such peroxidized phospholipidic species is compatible with the observed molecular weight increase by up to 1.2 kDa (Girón-Calle et al., 1994). From this point of view, exposure of an additional cysteine residue in the m-state as described by Klingenberg and co-workers (Aquila et al., 1982; Aquila & Klingenberg, 1982) could be enough to explain the conformational specificity of the ANT modification. It is known that chemical modification of this residue locks the protein in the m-state, preventing any translocase activity (Aquila et al., 1982; Aquila & Klingenberg, 1982). According to the work of Majima and coworkers (1993, 1994), this residue probably is Cys^{56}, located in the first of the three loops of the protein that are exposed to the mitochondrial matrix (Klingenberg, 1989; Klingenberg & Nelson, 1994). This is the only cysteine residue that is protected from alkylation in submitochondrial particles in the presence of CAT, but not of BKA (Majima et al., 1994). Alkylation of Cys^{56} inhibits ADP transport (Majima et al., 1993). Interestingly, it has been shown that binding of cardiolipin, which is required for ADP/ATP transport, is mediated in yeast by Cys^{73}, the residue homologous to Cys^{56} (Hoffmann et al., 1994).

An important consequence of the conformation dependence of ANT modification is that the susceptibility of this protein to peroxidative attack will be determined by the metabolic situation of the cell. Thus, peroxidative ANT modification will be mediated by the effect that key metabolites such as adenine nucleotides, fatty acyl-CoA, and oxidizable substrates have on the orientation of ANT (Lê-Quôc & Lê-Quôc, 1989; Vignais, 1976). The peroxidative alteration of ANT is of special interest because this protein is vital for the generation of energy in the cell. Ischemia-reperfusion injury involves the depletion of adenine nucleotides and damage to mitochondria (Reimer & Jennings, 1992). Although the primary cause for the energy imbalance in injured myocytes is subject of debate, damage to ANT could be involved. There is evidence showing a reduction of ANT activity in ischemic myocardium (Duan & Kamazyn, 1988; Regitz et al., 1984; Shug et al., 1975). If the conformation dependence shown here occurs due to peroxidative stress during episodes of ischemia-reperfusion *in vivo*, it would constitute a critical factor for understanding the process of injury to the myocyte and for developing therapeutic treatments.

Finally, it is interesting to consider our results in the context of the nonspecific permeability transition that is induced in mitochondria by treatment with calcium and prooxidants [Valle et al., 1993; reviewed by Gunter and Pfeiffer (1990), Halestrap et al. (1993), and Zoratti and Szabò, (1995)]. This involves loss of sulfhydryl groups in membrane-bound proteins and depends on the translocational state adopted by ANT. It has, in fact, been proposed that ANT might constitute, or at least be part of, the proteinaceous pore responsible for the loss of the permeability barrier itself (Halestrap et al., 1993; Halestrap & Davidson, 1990; Lê Quôc & Lê Quôc, 1988; Novgorodov et al., 1994; Zoratti & Szabò, 1995).

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